Dentritic cells and the battle against arthritis
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Chapter 3

Immunomodulatory dendritic cells inhibit Th1-responses and arthritis via different mechanisms

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Immunomodulatory Dendritic Cells Inhibit Th1 Responses and Arthritis via Different Mechanisms[1]

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Dendritic cells (DCs) are professional APCs which have the unique ability to present both foreign and self-Ags to T cells and steer the outcome of immune responses. Because of these characteristics, DCs are attractive vehicles for the delivery of therapeutic vaccines. Fully matured DCs are relatively well-defined and even used in clinical trials in cancer. DCs also have the potential to influence the outcome of autoimmunity by modulating the underlying autoimmune response. To gain a better appreciation of the abilities and mechanisms by which immunomodulatory DCs influence the outcome of T cell responses, we studied several immunomodulatory DCs (TNF-, IL-10-, or dexamethasone-stimulated bone marrow-derived DCs) side by side for their ability to modulate T cell responses and autoimmune diseases. Our data show that these differentially modulated DCs display a different composition of molecules involved in T cell activation. Although, all DC subsets analyzed were able to inhibit the induction of collagen-induced arthritis, the modulation of the underlying immune response was different. Vaccination with TNF- or IL-10-modulated DCs altered the Th1/Th2 balance as evidenced by the induction of IL-5- and IL-10-secreting T cells and the concomitant reduction of the IgG2a-IgG1 ratio against the immunizing Ag. In contrast, DCs modulated with dexamethasone did not affect the ratio of IL-5-producing vs IFN-γ-producing T cells and tended to affect the Ab response in a nonspecific manner. These data indicate that distinct mechanisms can be used by distinct DC subsets to change the outcome of autoimmunity. *The Journal of Immunology,* 2007, 179: 1506–1515.

The immune system is constantly confronted with Ags and proteins that have not been encountered previously. Such new Ags can be found intracellularly (for example, viruses and certain bacteria) or extracellularly (for example, protozoa and helminthes). To combat these different sets of pathogens, the immune system has to mount different immune responses. Th1 cells and CTLs will be effective against intracellular pathogens but not against extracellular pathogens, whereas Th2 cells and IgE-secreting B cells are crucial in the defense against extracellular parasites. Moreover, the immune system is also facing a third set of Ags on a regular basis to which no "conventional" immune reaction (i.e., Th1/Th2 reaction) should be mounted as this would be harmful to the host. These novel Ags can enter the organism via food intake or via the airway epithelium. Similarly, also during pregnancy no "conventional" immune reaction against the unborn child is desired.

To drive the immune response into the required direction, the immune system is equipped with a highly plastic cell type, the dendritic cell (DC)[4] (1). DCs are often divided in two populations, immature and mature. Immature DCs are capable of Ag uptake. After an activation stimulus (for example, TLR triggering or CD40-CD40L signaling), DCs mature as evidenced by an up-regulation of MHC and costimulatory molecules. These mature DCs are no longer capable of Ag uptake but are now endowed with the capacity to initiate Ag-specific T cell responses. In contrast, immature DCs are thought to induce Ag-specific tolerance via the induction of regulatory T cells or via the deletion of Ag-specific "conventional" T cells. Thus, DCs play a pivotal role in orchestrating the immune response, as the activation status of DCs imposes an important regulatory control on the induction and tolerization of immune responses against self- and nonself-Ags.

In this study, the capacity of DCs as protective vaccine for collagen-induced arthritis (CIA) was investigated. CIA is a well-defined animal model for rheumatoid arthritis (RA) in humans. Although this mouse model is certainly not similar to RA, many characteristics, such as the systemic nature of the arthritis, the production of autoantibodies, the chronic and destructive inflammation of joints, as well as the association with the MHC class II molecules, highly resemble important features of RA (2). Consequently, it is presumed that the most prominent immunologic and inflammatory mechanisms that are operative in RA are also present in CIA.

CIA is induced through immunization with bovine type II collagen (CII) emulsified in CFA. Endogenous DCs will present the

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[4] Abbreviations used in this paper: DC, dendritic cell; CIA, collagen-induced arthritis; RA, rheumatoid arthritis; CII, type II collagen; Dex, dexamethasone; PBA, PBS containing 0.5% BSA, 0.02% azide; PBA-sap, PBA containing 0.1% saponin; HA, influenza virus A; Treg, regulatory T cell; ACPA, Ab against citrullinated protein.

Table I. *Primers and probes for RT-PCR*

	Sequences
Delta 4	
Sense	5'-CAG AGA CTT CGC CAG GAA AC-3'
Antisense	5'-CTC TCC GTG CAA GAA TGG AT-3'
Jagged 1	
Sense	5'-GTG AAG ACC AAG TGC ACA TC-3'
Antisense	5'-GGA TAA ACA CCA GCA GAA AG-3'
Jagged 2	
Sense	5'-GTC CTT CCC ACA TGG GAG TT-3'
Antisense	5'-ACC GAG GTC AAG GTG GAA A-3'
β-actin	
Sense	5'-AGA GGG AAA TCG TGC GTG AC-3'
Antisense	5'-CAA TAG TGA TGA CCT GGC CGT-3'

CII to naive T cells, which will differentiate mainly into IFN-γ-producing Th1 cells. Likewise, under the influence of the CII-specific Th1 cells, B cells get activated and start to produce CII-specific Abs. These anti-CII Abs are primarily of the IgG2a isotype (Th1-associated IgG isotype). This IgG isotype is shown to be superior in complement activation (3) and is able to interact efficiently with FcγR (4, 5). The CII-specific Abs are shown to be crucial and sufficient for disease induction (6, 7) and therefore CIA can be considered a systemic, B cell-dependent autoimmune disease.

To design a cellular vaccine for such a systemic, B cell-mediated autoimmune disease, the immune response against CII should be skewed toward a Th2-like phenotype or, alternatively, a "regulatory" phenotype to modulate the induction of pathogenic CII-specific Th1 cells and thereby the evolving B cell reaction (less anti-CII Abs, especially of the IgG2a isotype). For this purpose, it is thought that the "environment" in which DCs are activated and obtain their Ag should not lead to "full maturation" of DCs, but rather support the emergence of a DC with an "immature" or "semimature" phenotype.

Several pharmacological agents such as TNF, IL-10, dexamethasone (Dex), vitamin D3, and vasoactive intestinal peptide have been used to modulate the immunomodulatory capacities of DCs. Of these, we selected three different methods to modulate DCs as these have consistently been shown to affect the outcome of adoptive immune responses (8–13). In this study, we have investigated these three types of "immunomodulatory" DCs and analyzed them "side by side" for their capacity to inhibit CIA. Although all three DC subsets were able to inhibit progression of arthritis, the underlying pathways induced by these DCs were different. These data indicate that the selection of the modulatory agent for DC-based immune intervention will depend on the mechanisms underlying the disease that is targeted.

Materials and Methods

Mice

DBA/1J and D0.11.10 (OVA-specific TCR-transgenic) mice were obtained from our own breeding colonies. BALB/CBYJico mice were purchased from Charles River Laboratories. Mice were kept at the Leiden University Medical Center animal facility and used at 8–10 wk of age. Experiments were performed in accordance with national legislation and under supervision of the Animal Experimental Committee of the University of Leiden.

Induction of CIA and evaluation of arthritis

CIA was induced in male DBA/1J mice (H-2q). Bovine CII protein (MD Biosciences) was dissolved in 0.1 M acetic acid solution overnight at 4°C at a concentration of 2 mg/ml. The dissolved CII (100 μg of CII/mouse) was emulsified with an equal volume of CFA (Difco) and 100 μl was injected s.c. into the base of the tail. This immunization was boosted 3 wk later with a s.c. injection of 100 μg of CII emulsified in IFA (Difco).

Development of clinical arthritis was followed through visual scoring of the animals based on the number of inflamed joints in each paw, starting 2 wk postimmunization and continuing until the end of the experiment. An extended scoring protocol ranging from 1 to 15 for each paw with a maximum score of 60 per mouse was used (14). The mice were examined three times per week. When a mouse had two paws with a maximum score of 15, it was sacrificed because of ethical constraints, as defined by the local ethical committee. The last score measured at the time of sacrifice was kept as the final arthritis score for that mouse.

Preparation of bone marrow-derived DCs

DCs were generated from bone marrow obtained from DBA/1J or BALB/c mice according to a previously described procedure (15). Briefly, bone marrow cells were cultured in GM-CSF containing medium (16). After 10 days of culture, DCs were harvested and used as immature DCs. DCs were activated with 500 U/ml TNF (Tebu-Bio) or with 1 μg/ml LPS (Sigma-Aldrich). When DCs were modulated with IL-10 (50 ng/ml; Tebu-Bio) or Dex (1 μM; Sigma-Aldrich) (17), IL-10 or Dex were added on day 9, 24 h before the addition of LPS.

Abs and FACS analysis

The following Abs were purchased from BD Pharmingen: FITC-labeled anti-CD11c, anti-CD86, anti-CD3ε; PE-labeled anti-CD40, anti-CD80, anti-class II (clone M5/115), anti-IFN-γ, anti-IL-5, anti-IL-10, anti-IgG1, and anti-IgG2b; PerCP-Cy5.5 and allophycocyanin-labeled anti-CD4. Allophycocyanin-labeled KJ1-26 (anti-D011.10 TCR) was derived from Caltag Laboratories. PE-conjugated anti-PD-L1 and anti-PD-L2 were purchased from eBioscience. For the detection of surface markers, DCs were incubated with the appropriate Abs for 15 min at 4°C in the dark. After three washing steps, the cells were fixed in 0.5% paraformaldehyde.

Table II. *Primers and probes for real-time PCR*

|	Sequences
Delta 4	
Sense	5'-AGG TGC CAC TTC GGT TAC ACA G-3'
Antisense	5'-CAA TCA CAC ACT CGT TCC TCT CTT C-3'
Probe	5'-TCG GTT ACA CAG TGA GAA GCC AGA-3'
Jagged 1	
Sense	5'-AGA AGT CAG AGT TCA GAG GCG TCC-3'
Antisense	5'-AGT AGA AGG CTG TCA CCA AGC AAC-3'
Probe	5'-CTG AAA AAC AGA ACA GAT TTC CTG GT-3'
β-actin	
Sense	5'-AGA GGG AAA TCG TGC GTG AC-3'
Antisense	5'-CAA TAG TGA TGA CCT GGC CGT-3'
GAPDH	
Sense	5'-ACT CCC ACT CTT CCA CCT TC-3'
Antisense	5'-CAC CAC CCT GTT GCT GTA G-3'
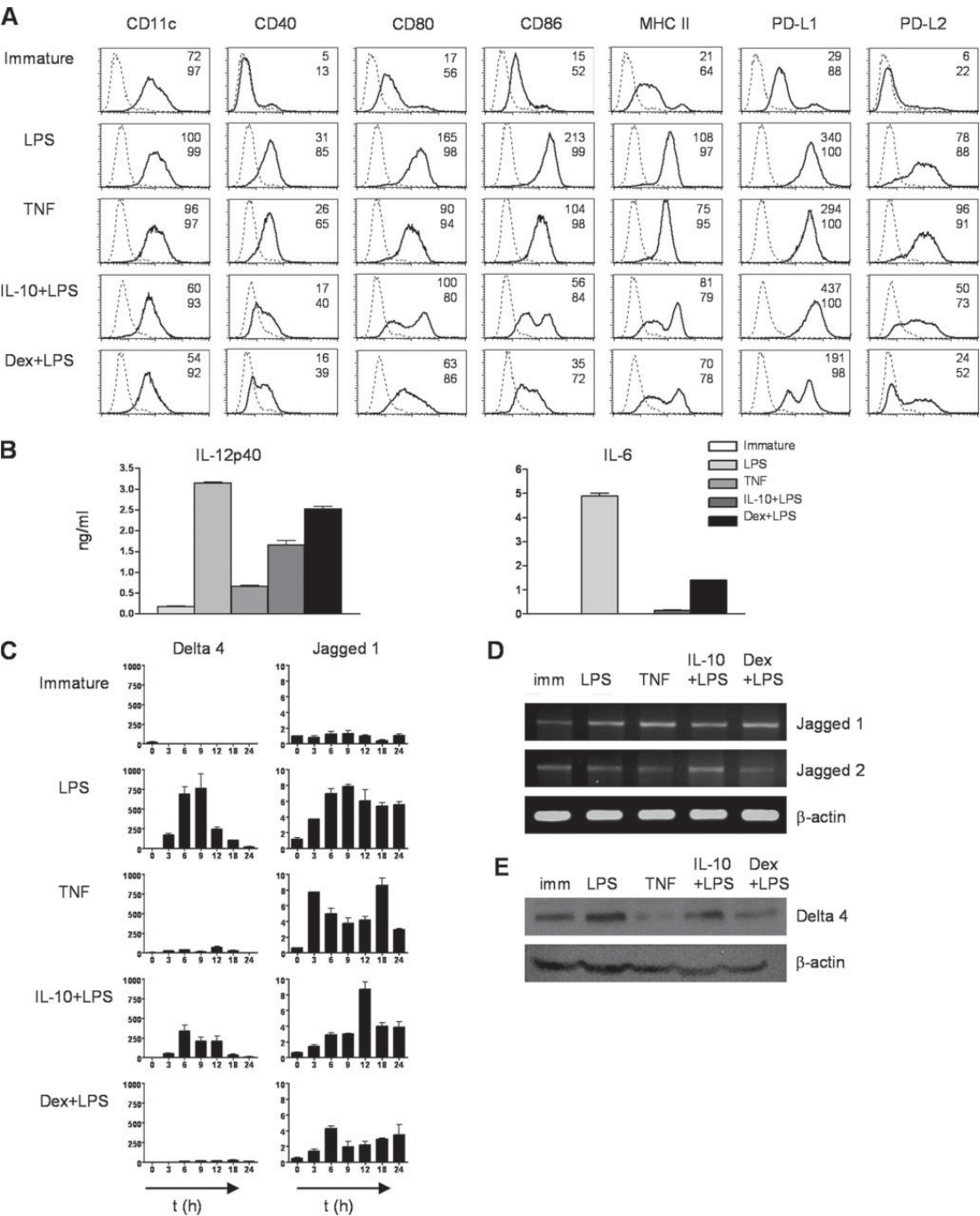
FIGURE 1. Phenotype and cytokine production of differentially modulated DCs. *A*, DCs were generated from DBA/1J mice. After 10 days of culture in GM-CSF-containing medium, the expression of CD11c and costimulatory molecules (thick lines) were measured by FACS. Thin lines show isotype controls. DCs stimulated with TNF, IL-10, or Dex were compared with nonactivated and LPS-activated DCs. Data from a representative experiment (of three experiments performed) are shown. Upper values represent mean fluorescent intensity, whereas lower values represent percentages of positive cells. *B*, IL-12p40 and IL-6 production was measured after an overnight culture of differentially modulated DCs. DCs stimulated with TNF, IL-10, or Dex were compared with nonactivated DCs and LPS-activated DCs. Data from a representative experiment (of three experiments performed) are shown. Values are means ± SEM. *C*, DCs were stimulated in time with different stimuli. RNA was isolated and quantitative PCR was performed on cDNA. Fold increase of Delta 4 and Jagged 1 relative to GAPDH in comparison with immature DCs is shown. Data from one representative experiment (of three experiments

For intracellular cytokine staining, cultured D011.10 CD4^{+} T cells were stimulated overnight with the appropriate DC. During the last 3 h of stimulation, 10 μg/ml brefeldin A (Sigma-Aldrich) was added. After this incubation step, cells were stained for cell-surface markers (20 min, on ice). Next, the cells were washed in PBS containing 0.5% BSA, 0.02% azide (PBA) and fixed in 4% paraformaldehyde for 5 min (all incubations were done on ice). Cells were washed in PBA and in PBA containing 0.1% saponin (PBA-sap) and thereafter permeabilized in PBA-sap supplemented with 10% FCS for 20 min. Staining was performed with Abs diluted in PBA-sap for 20 min, washed with PBA, and fixed in 0.5% paraformaldehyde. Data acquisition and analysis were performed on a FACSCalibur using CellQuestPro software (BD Biosciences).

Cytokine measurement by ELISA

Supernatants of nonactivated DCs or activated DCs were used. DCs were either stimulated with TNF (500 U/ml) for 24 h or with LPS (1 μg/ml) for 24 h. The modulation with either IL-10 (50 ng/ml) or Dex (1 μM) was done 24 h earlier, and after these 24h, LPS (1 μg/ml) was additionally added for 24 h to the DC cultures, and supernatant was taken from these cultures. These supernatants were tested for IFN-γ, IL-4, IL-10, and IL-12p40 content using a standard sandwich ELISA. All coating- and detection Abs were purchased from BD Pharmingen. Streptavidin-HRP (Sanquin) and ABTS (Sigma-Aldrich) were used as enzyme and substrate, respectively.

Cytokine measurement by BD CBA flexset

Supernatants were obtained and described for cytokine measurements by ELISA. The supernatants were tested for IL-12p70 and IL-6 contents according to manufacturer's protocol. Data acquisition and analysis were performed on a FACS LSRII using FACSDiva software (BD Biosciences).

Notch-ligand expression measured by RT-PCR

Total RNA was isolated from DCs using the TRIzol reagent (Invitrogen Life Technologies) and samples were incubated with RNase-free DNase I (Invitrogen Life Technologies) at room temperature for 15 min to avoid amplification/detection of contaminating genomic DNA. One microgram of total RNA was reversely transcribed by using transcriptase enzyme and specific cDNA was amplified using primers, which are depicted in Table I.

Delta 4 and Jagged 1 expression measured by real-time PCR

Total RNA was isolated as mentioned above. Equal amounts RNA equivalents of cDNA were used in each quantitative PCR amplification, run in duplicate on the same plate. Detection of the PCR product was conducted by the iCycler real-time PCR detection system (Bio-Rad) using the SYBR green mix for the reference genes and TaqMan probes for the genes of interest. Gene expression levels were normalized using the two most stable reference genes selected by GeNorm (Bio-Rad). Primers and probes are depicted in Table II.

Protein expression measurements using Western blot

DCs were stimulated as described above. Cells were lysed in the presence of Ral lysis buffer, containing 1% Igepal CA-630 (Sigma-Aldrich). Different numbers of DC were loaded 1:1 with sample buffer (Bio-Rad) and placed on a 8% acrylamide-bis gel. Thereafter, the gel was blotted on a PWPF membrane (Millipore). Membranes were probed with Delta 4 polyclonal Ab (Santa Cruz Biotechnology) and anti-β-actin (Sigma-Aldrich) Ab. Specific bands were visualized by ECL staining (Amersham Biosciences).

Induction of OVA-specific T cell responses in vitro

Spleen cells were isolated from D011.10 mice and purification by nylon wool was performed. T cells were stimulated with OVA[327–339]-loaded DCs (with the appropriate stimulus) and cultured in IMDM (Cambrex Bioscience) containing 8% heat-inactivated FCS (Bodinco), 100 U/ml penicillin, 2 mM L-glutamine, and 20 μM 2-ME. T cells were cultured with DCs in a ratio of 10:1 and restimulated on day 7 after stimulation

(for three times). Cells were then harvested, counted, and stimulated overnight with Ag-loaded DCs (OVA[327–339], or as control HA[139–151]) or with 5 μg/ml Con A (Sigma-Aldrich) and intracellular cytokine staining was performed.

Treatment of mice with DCs

For in vivo experiments, DCs were stimulated with TNF (500 U/ml) for 4 h or with LPS (1 μg/ml) for 4 h. The modulation with either IL-10 (50 ng/ml) or Dex (1 μM) was done 24 h earlier, and after these 24 h, LPS (1 μg/ml) was additionally added for 4 h to these DC cultures. Together with the TNF or LPS, the appropriate peptide (CII, OVA, Dengue, or influenza virus A (HA)) (10 μg/ml) was also added. After the incubation, DCs were harvested, washed twice, and counted. Mice were given i.v. injections (on days −7, −5, and −3 before immunization, unless described otherwise) of 2.5×10^6 DCs.

Measurement of Ag-specific Abs in serum

Abs were measured using a standard sandwich ELISA. Immuno-Maxisorp 96-well plates (Nunc) were coated overnight at 4°C with 2 μg/ml protein (bovine CII, murine CII, or OVA). After washing with PBS-0.5% Tween 20, plates were blocked with 10% milk for 2 h at 4°C. Serially diluted mouse serum was then incubated overnight at 4°C on the washed plates. Plates were subsequently treated with one of the following detection Abs: HRP-conjugated anti-mouse IgGAM, IgG1, or IgG2a (Southern Biotechnology Associates). Detection was performed using tetramethylbenzidine (Sigma-Aldrich) as substrate.

Statistical analysis

Differences in disease severity and in Ab production were analyzed with the nonparametric Mann-Whitney U test. Values of $p < 0.05$ with a 95% confidence interval were considered significant.

Results

Influence of different stimuli on the maturation of DCs

DCs are thought to play a critical role in setting the immune response against (self-) Ags, mediating either immunity or tolerance. Because levels of costimulatory molecules, like CD40, B7.1, B7.2, PD-L1, and PD-L2, and the secretion of proinflammatory cytokines, like IL-12 and IL-6, are thought to play a pivotal role in these processes, we investigated the expression levels of these molecules displayed by different subsets of DCs.

DCs were generated from bone marrow-derived cells, obtained from a DBA/1J mouse. After 10 days of culture in GM-CSF-containing medium, cells were analyzed for surface molecule expression. In Fig. 1A, it is shown that all cells expressed CD11c. To study the response of DCs to various maturation stimuli, DCs were treated with LPS (as control), TNF, IL-10 plus LPS, and Dex plus LPS. Untreated DCs were used as immature control. After 24 h, cells were harvested and analyzed by FACS for the expression of costimulatory molecules and MHC class II (Fig. 1A). TNF-stimulated DCs displayed a "semimature" phenotype, as they express more costimulatory molecules compared with immature DCs, but less than LPS-activated DCs as also published before (8, 18). IL-10 was not completely able to suppress the induction of these molecules by LPS, as the IL-10-treated DCs have a comparable phenotype as fully matured DCs. Nonetheless, exposure to Dex inhibits the maturation of DCs by LPS as a reduced up-regulation of CD40, B7.1, B7.2, PD-L1, PD-L2, and MHC class II molecules was observed. In contrast, pretreatment with Dex only marginally affected the ability of DCs to produce IL-12p40 and IL-6 after exposure to LPS, whereas IL-10 treatment was more effective in

performed) are shown. Values are means ± SEM. *D*, DCs were stimulated for 6 h with different stimuli. RNA was isolated and transcribed to cDNA. The expression of cDNA for Jagged 1 and 2 and β-actin as control is depicted. Data from one representative experiment (of five experiments performed) are shown. *E*, DCs were stimulated for 24 h with different stimuli and protein expression of Delta 4 and β-actin was measured by Western blot. Data from one representative experiment (of three experiments performed) are shown.

inhibiting both IL-12p40 and IL-6 production. TNF-modulated DCs only produced modest amounts of IL-12p40 and no IL-6 was detected (Fig. 1*B*). We were not able to detect IFN-γ, IL-4, or IL-10 production by these DC populations by standard ELISA, indicating that the production of these cytokines is low or absent. Likewise, only modest amounts of IL-12p70 were detected (data not shown).

Expression of Notch ligands on differentially modulated DCs

Recently, it has been shown that the Notch ligands Delta 4 and Jagged 1 are important in setting the outcome of immune reactivity. The expression of Delta 4 on DCs is associated with Th1 activation, whereas Jagged 1 expression is involved in the induction of Th2 cells and have been implicated in the emergence of regulatory T cells (19, 20). To study whether LPS and/or TNF treatment does lead to the expression of either one of these Notch ligands, and to study whether this expression is influenced by IL-10 or Dex treatment, DCs were stimulated in time with the different stimulations and fold increase of both Delta 4 and Jagged 1 were measured by real-time PCR. All DCs expressed Jagged 1 and 2, although the level of expression is not significantly changed in time upon stimulation (Fig. 1, *C* and *D*). Remarkably, substantial differences were found with respect to Delta 4 expression rapidly after exposure to LPS. Likewise, its expression is also quickly down-regulated, leading to basal mRNA expression levels 24 h after stimulation. This increase in Delta 4 expression is abrogated when DCs were activated in the presence of Dex, whereas IL-10 treatment led to a diminished induction of Delta 4 expression following LPS stimulation. In contrast, TNF stimulation did not induce up-regulation of Delta 4. These results were confirmed by Western blotting (Fig. 1*E*) indicating that also the protein levels of Delta 4 are influenced by the different DC stimulations.

Together, these data indicate that different treatments affect activation of DCs differentially, which is likely to be of relevance for the ability of DCs to affect and steer the outcome of immune reactivity following vaccination.

Inhibition of CIA after vaccination with "immunomodulatory" DCs

The data presented above imply that the differentially modulated DCs will have distinct immunomodulatory capacities, which could affect their ability to modulate the outcome of autoimmune diseases. Therefore, we wished to compare, side by side, the effects of vaccination with differentially modulated DCs on the outcome of CIA. To this end, TNF, IL-10, Dex, and LPS (as control) stimulated DCs were pulsed with bovine CII-peptide 256–270, the immunodominant epitope for CII-directed $CD4^+$ T cells. These DCs were then injected i.v. on days 7, 5, and 3 before CIA induction. As previously described, LPS-activated DCs did not result in a significant decrease in severity of the disease (Ref. 18 and Fig. 2*A*), whereas DCs exposed to TNF were able to inhibit disease progression. When the latter effects were compared with the effects installed by vaccination with IL-10- or Dex-modulated DCs, we found that all three DC subsets were able to decrease the disease severity after vaccination as compared with control mice, which were only immunized with CII/CFA (Fig. 2*B*). The decrease in disease severity is Ag specific for TNF- and IL-10-modulated DCs, as vaccination with DCs pulsed with a control peptide did not result in a significant decrease in disease severity (Ref. 18 and Fig. 2*C*). For Dex-stimulated DCs, there was no significant difference observed in disease severity after vaccination with DCs loaded with the CII peptide or a control peptide (data not shown), suggesting that a non-Ag-specific mechanism is involved in the ef-
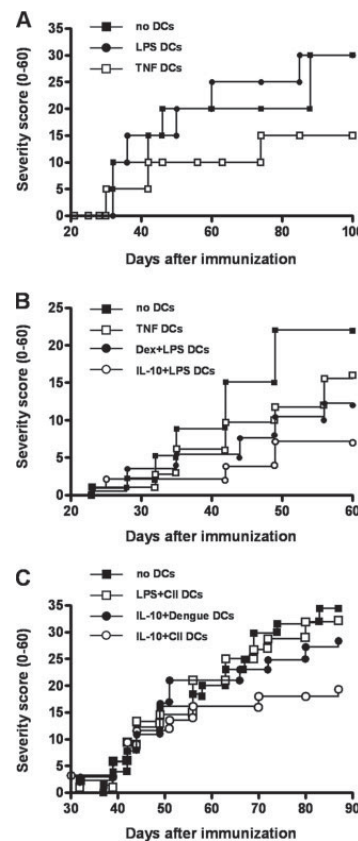

FIGURE 2. Reduced severity of CIA after vaccination with DCs, stimulated with TNF, IL-10, or Dex. Modulated DCs were pulsed with $CII^{256-270}$-peptide and 2.5×10^6 cells were injected i.v. at three time points (days −7, −5, −3) before immunization with CII protein in CFA. Control mice were not treated with DCs, but were only immunized with CII/CFA. *A*, Mean arthritis severity score in each group over time. Mice treated with TNF-modulated DCs had a significant lower disease severity compared with nontreated individuals ($p < 0.05$). Vaccination with LPS-activated DCs had no effect on disease severity compared with nonvaccinated mice. Data from one representative experiment (of three experiments performed) ($n = 13$ mice/group) are shown. *B*, Mean arthritis severity score in each group over time. Mice treated with either one of the differentially modulated DCs had a lower arthritis severity score compared with nonvaccinated mice (TNF DCs: $p = 0.0005$, IL-10 and Dex DCs: $p < 0.0001$). Data from one representative experiment (of two experiments performed) ($n = 13$ mice/group) are shown. *C*, Ag specificity of IL-10-modulated DCs. Mice were treated with IL-10-modulated DCs either pulsed with $CII^{256-270}$-peptide or $Dengue^{59-78}$-control peptide. The mean arthritis severity score is shown for each group in time. Only mice treated with IL-10-modulated DCs pulsed with the CII peptide showed a significant decrease in disease severity compared with nonvaccinated mice ($p < 0.0001$) and compared with mice vaccinated with IL-10-modulated DCs pulsed with the control peptide ($p = 0.0002$). Mice vaccinated with IL-10-modulated DCs pulsed with the control peptide did not show a significant decrease in disease severity compared with nonvaccinated mice ($p = 0.492$) Data from one experiment (of two experiments performed) are shown ($n = 9$ mice/group).

fects, initiated by Dex-modulated DCs. These data indicate that all immunomodulatory DCs can inhibit or decrease a Th1-associated (auto) immune response.
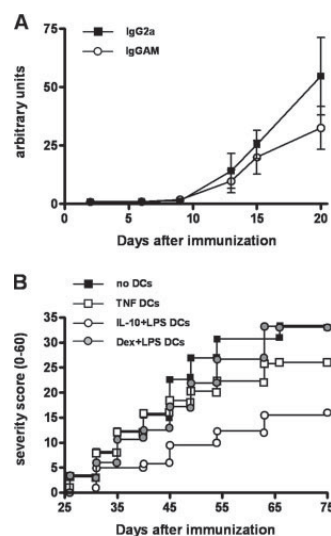
FIGURE 3. IL-10-modulated DCs are also able to reduce disease severity in the presence of an ongoing pathogenic immune reaction. Mice were immunized with CII/CFA to induce arthritis. *A*, On several days after immunization with CII/CFA, blood samples were collected to measure anti-murine CII Ab titers. Data from one representative experiment (of two experiments performed) ($n = 5$ mice/group) are shown. Values are means $\pm$ SEM. *B*, On days 19, 21, and 23 after immunization with CII/CFA, mice were treated with TNF-, IL-10-, or Dex-modulated DCs (2.5×10^6 cells/day, i.v.). Control mice were not treated with DCs, but were only immunized with CII/CFA. Mean arthritis severity score in each group over time is shown. Mice treated with TNF- or IL-10-modulated DCs had a significant lower arthritis severity score compared with nontreated mice ($p = 0.02$ and $p < 0.0001$, respectively). Data from one representative experiment ($n = 10$ mice/group) are shown.

FIGURE 4. Altered CII-specific IgG2a-IgG1 ratio after vaccination with either one of the immunomodulatory DCs. Modulated DCs were pulsed with CII[256–270] peptide and 2.5×10^6 cells were injected i.v. at three time points (days -7, -5, -3) before immunization with CII protein in CFA. Control mice were not treated with DCs, but were only immunized with CII/CFA. *A*, Total Ig (IgA, IgG and IgM) Abs against murine CII were measured in sera on day 36 after immunization with CII/CFA. Data from one representative experiment (of two experiments performed) ($n = 13$ mice/group) are shown. Values are means $\pm$ SEM. *B*, IgG2a-IgG1 ratio (directed against anti-murine CII) in mice vaccinated with differentially modulated DCs were compared with nontreated mice. Abs were measured on day 36 after immunization in sera of mice. Mice treated with either TNF- or IL-10-modulated DCs displayed a lower IgG2a-IgG1 ratio compared with nonvaccinated mice (TNF DCs: $p = 0.05$, IL-10 DCs: $p = 0.04$), whereas the effects observed after vaccination with Dex-modulated DCs were not statistically significant ($p = 0.16$). Data from one representative experiment (of two experiments performed) ($n = 13$ mice/group) are shown. Values are means $\pm$ SEM.

Vaccination with IL-10 modulated DCs inhibits progression of CIA in the presence of an established CII-specific immune response

The data described above indicate that DC vaccination could modulate the outcome of CIA in case the immune system is not triggered yet by the disease causing Ag (i.e., bovine CII). To investigate whether DC vaccination could also influence the outcome of arthritis in the presence of an established CII-specific immune reaction, as evidenced by the presence of CII-specific Abs, we vaccinated mice with TNF-, IL-10-, or Dex-modulated DCs 3 wk after the first challenge with bovine CII in CFA. At this point, CII-specific Abs against both bovine (data not shown) and mouse CII are readily detectable (Fig. 3*A*).

Also in this setting, vaccination with DCs resulted in an inhibition of disease progression as became apparent by lower severity scores in the mice that received IL-10-modulated DCs (Fig. 3*B*). Treatment with Dex-modulated DCs could not inhibit disease severity as compared with nonvaccinated mice, whereas TNF-stimulated DCs were able to modestly suppress disease severity. However, the effects installed by TNF-stimulated DCs were less pronounced as compared with the effects mediated by IL-10-modulated DCs (Fig. 3*B*). Together, these data indicate that DCs modulated by IL-10 cannot only be used to prevent CIA in a prophylactic setting, but also at a time the pathogenic immune response is already ongoing.
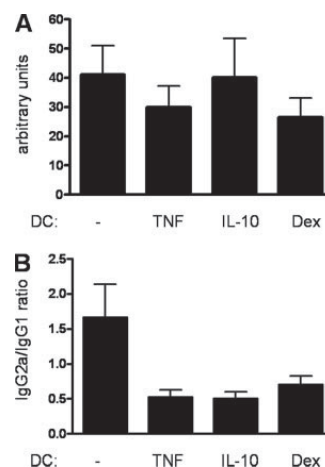
Altered CII-specific IgG2a-IgG1 ratio after vaccination with immunomodulatory DCs

The data presented above show a protective effect induced by vaccination with either one of the immunomodulatory DCs. Because CII-specific Abs are crucial for disease induction (21), we next investigated whether CII-specific B cell responses were affected by vaccination with immunomodulatory DCs. Serum was collected at different time points after disease induction and Ab titers were measured by ELISA.

As shown in Fig. 4*A*, titers of CII-specific Abs (IgA, IgG, and IgM, directed against murine CII) were comparable between all groups. However, after vaccination with TNF- and IL-10-treated DCs, a decrease in IgG2a-IgG1 ratio of CII-specific Ab titers was observed (Fig. 4*B*). A tendency for a similar alteration in isotype usage of CII-specific Abs was observed after vaccination with Dex-treated DCs. Together, these data indicate that treatment with CII-pulsed immunomodulatory DCs can modulate the outcome of B cell immunity against CII, as evidenced by a decrease in the ratio between IgG2a and IgG1 Ab titers.

The effects mediated by vaccination with immunomodulatory DCs do not crucially depend on the strain of mice or the immunizing Ag

The data described above show that vaccination with all selected types of immunomodulatory DCs affect both the outcome of CIA as well as the underlying CII-specific B cell response. To analyze
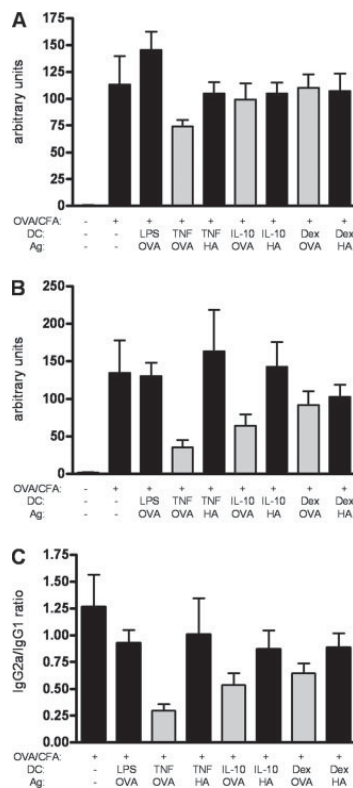
FIGURE 5. TNF- and IL-10-modulated DCs show a decrease in IgG2a Ab titers in an Ag-specific manner, whereas the effects induced by Dex-stimulated DCs are not Ag specific. DCs were stimulated either with TNF, IL-10, or Dex. All these DCs were pulsed with OVA17 peptide ($OVA^{327–339}$) or with a control peptide from influenza A ($HA^{139–151}$). Cells (2.5×10^6) were injected i.v. at three time points (days -7, -5, and -3) before immunization with OVA/CFA. Control mice were not treated with DCs, but were only immunized with OVA/CFA. *A*, Total Ig (IgA, IgG, and IgM) Abs against OVA were measured in sera on day 36 after immunization. All data were obtained from two experiments ($n = 13$ mice/group), except the data for the Dex DC-treated groups (including the controls "no DC" and "LPS+ OVA DC") which were derived from three experiments ($n = 25$ mice/ group). Values are means $\pm$ SEM. *B*, Anti-OVA IgG2a Ab titers were measured in mice treated with differentially modulated DCs, nontreated mice, or mice treated with fully mature DCs. Abs were measured on day 36 after immunization in sera of mice. Mice vaccinated with TNF-stimulated, OVA17-pulsed DCs displayed significant lower IgG2a Ab titers compared with nonvaccinated mice ($p = 0.02$), compared with mice vaccinated with LPS-activated, OVA17-pulsed DCs ($p < 0.0001$) as well as compared with mice that were vaccinated with TNF-stimulated, HA-pulsed DCs ($p = 0.002$). Likewise, mice vaccinated with IL-10-stimulated, OVA17-pulsed DCs had significant lower IgG2a Ab titers compared with nonvaccinated mice ($p < 0.0001$), compared with mice vaccinated with LPS-activated, OVA17-pulsed DCs ($p = 0.008$) as well as compared with mice vaccinated with IL-10-stimulated, HA-pulsed DCs ($p = 0.018$). There was no significance obtained after vaccination with Dex-modulated, OVA peptide-pulsed DCs compared with nonvaccinated mice ($p = 0.64$). Similarly, no significant effects were observed when these DC-treated mice were compared with mice vaccinated with LPS-activated, OVA17-pulsed DCs ($p = 0.07$) or compared with mice vaccinated with Dex-modulated, HA-pulsed DCs ($p = 0.25$). Values are means $\pm$ SEM. *C*, IgG2a-IgG1 ratio in mice vaccinated with differentially modulated DCs were compared with non-treated mice. Abs were measured on day 36 after immunization in sera of mice. Mice vaccinated with TNF-stimulated, OVA17-pulsed DCs

whether these effects also extent to other Ags and other mouse strains as well as to obtain a better appreciation of the underlying mechanism, we wished to investigate the effects of vaccination with TNF-, IL-10-, and Dex-treated DCs on the OVA response in BALB/c mice. Therefore, TNF, IL-10, or Dex DCs were loaded with the immunodominant $CD4^+$ T cell epitope from OVA ($OVA^{327–339}$) or with the immunodominant epitope from influenza virus A ($HA^{139–151}$) as control. These cells were i.v. injected on days 7, 5, and 3 before immunization with OVA emulsified in CFA. At several time points after immunization, blood samples were collected to measure OVA-specific Abs. Similar to the findings described for CII, there were no apparent differences in total Ab titers (IgG, IgA, and IgM) directed against OVA (Fig. 5*A*). In the same way, there is a preferential decrease in IgG2a Ab titers after vaccination with immunomodulatory DCs (Fig. 5*B*). Intriguingly, vaccination with Dex-modulated DCs, loaded with a control Ag also tended to decrease IgG2a responses after immunization with OVA/CFA, indicating that the effects installed by Dex-stimulated DCs are not Ag specific. Together, these data indicate that the findings observed in the DBA/1 model can be extended to another strain of mice and another immunizing Ag. Furthermore, they suggest that Dex-modulated DCs also affect the immune response in an Ag-nonspecific manner.

Different cytokine-production profiles are induced in T cells exposed to different immunomodulatory DCs

The data described above indicate a specific decrease in the titers of IgG2a Abs against both CII and OVA after vaccination with TNF- or IL-10-modulated DCs. Although immunomodulatory effects of these three types of DCs have never been compared side by side, it has been indicated that all types of DCs induce IL-10-producing "regulatory" T cells (8–10, 22). To gain a better appreciation of the modulation of Ag-specific T cell responses that orchestrates the outcome of the B cell response, we set out to study the phenotype of the T cells induced after the different DC stimulations. Therefore, OVA-specific D011.10 T cells were stimulated with the differentially modulated DCs. After 3 wk of culture, polarized T cells were stimulated with Con A (Fig. 6*A*) to ensure that all T cells were exposed to the same stimulus used for readout. Alternatively, T cells were stimulated with LPS matured, OVA peptide-pulsed DCs for readout, yielding similar results (data not shown).

A clear decrease was observed in IFN-γ-producing OVA-specific T cells when these T cells have been cultured for 3 wk with TNF- or IL-10-modulated DCs compared with T cells that have been stimulated with LPS-activated DCs. Stimulation with TNF-modulated DCs resulted in a relative increase in both IL-5- and

displayed significant lower IgG2a-IgG1 ratio compared with nonvaccinated mice ($p = 0.002$), compared with mice vaccinated with LPS-activated, OVA17-pulsed DCs ($p < 0.0001$) as well as compared with mice which were vaccinated with TNF-stimulated, HA-pulsed DCs ($p = 0.006$). Likewise, mice vaccinated with IL-10-stimulated, OVA17-pulsed DCs had significantly lower IgG2a Ab titers compared with nonvaccinated mice ($p = 0.03$), compared with mice vaccinated with LPS-activated, OVA17-pulsed DCs ($p = 0.01$). There was no significance obtained with vaccination with Dex-modulated, OVA-peptide pulsed DCs compared with non-vaccinated mice ($p = 0.09$). Similarly, no significant effects were observed when these DC-treated mice were compared with mice vaccinated with LPS-activated, OVA17-pulsed DCs ($p = 0.07$) or compared with mice vaccinated with Dex-modulated, HA-pulsed DCs ($p = 0.07$). Values are means $\pm$ SEM.

FIGURE 6. The relative proportion of IFN-γ-, IL-5-, and IL-10-producing T cells differs between T cell populations that are induced by the different immunomodulatory DCs. D011.10 T cells were cultured constitutively for 3 wk with either one of the DC subsets as described in *Materials and Methods*. After 3 wk cytokine production (IFN-γ, IL-5, and IL-10) was measured by intracellular cytokine staining after overnight stimulation with Con A. *A*, Data from one representative experiment (of five experiments performed) are shown. *B*, To gain an indication of the relative proportions of cytokine-producing T cells by stimulation with the differentially modulated DCs, the ratios between the different cytokines produced by the T cells are depicted. *C*, D0.11.10 T cells cultured constitutively for 3 wk with Dex-modulated DCs were stimulated with LPS-activated or Dex-modulated DCs as readout for cytokine production. Whereas stimulation with LPS-matured, OVA-pulsed DCs had similar results as observed after activation with Con A for readout, the ratio of IL-10 over IFN-γ and IL-10 over IL-5-producing T cells increased further when OVA-loaded, Dex-modulated DCs were used for readout. Data from one representative experiment (of five experiments performed) are shown.
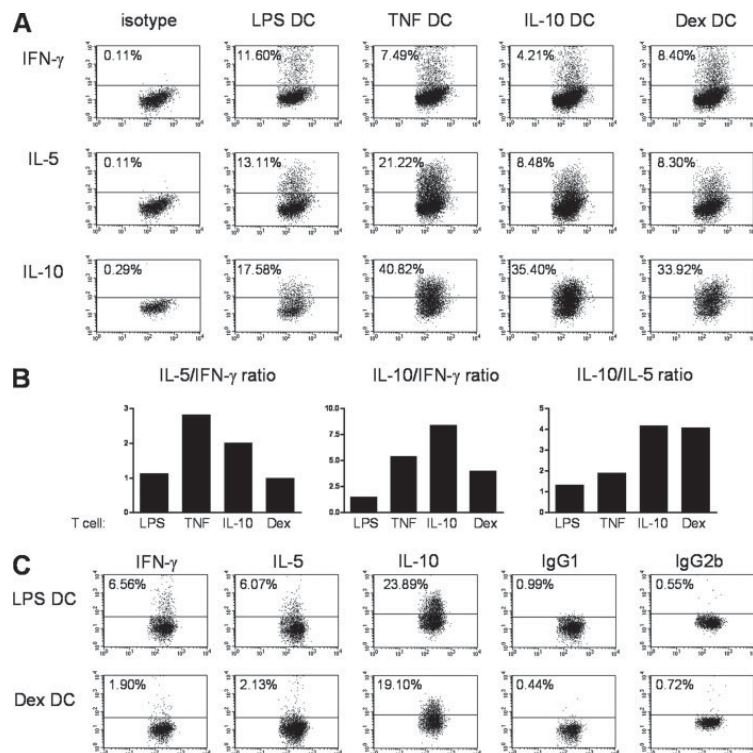
IL-10-producing T cells as also evidenced by an enhanced IL-5-IFN-γ and IL-10-IFN-γ ratios. Likewise, stimulation with IL-10-treated DCs readily results in a relative increase in IL-5- and IL-10-producing T cells over IFN-γ-producing T cells (Fig. 6*B*). These data indicate the induction of T cells with a Th2-like phenotype after stimulation with TNF- or IL-10-modulated DCs, which is in line with the decrease in IgG2a-IgG1 ratio. Intriguingly, T cell cultures stimulated with Dex-modulated DCs display the same ratio of IL-5-IFN-γ-producing T cells as compared with T cell cultures stimulated with LPS-activated DCs. These date indicate that no relative increase of IL-5-producing T cells is induced by Dex-modulated DCs. In contrast, an increase in IL-10- over IFN-γ-producing T cells, as well as an increase in IL-10- over IL-5-producing T cells was observed. As with Con A stimulation, the relative increase in IL-10 production was also observed when these T cell cultures were stimulated with LPS-activated DCs for readout, but became even more pronounced when T cells stimulated with Dex-modulated DCs were exposed to Dex-modulated DCs for readout (Fig. 6*C*) (with IL-10-IFN-γ and IL-10-IL-5 ratios of, respectively, 10 and 9). Together, these data indicate that, despite similar effects on the ability to modulate the outcome of CIA, Dex-modulated DCs induce a different T cell cytokine-production bias compared with TNF- and IL-10-modulated DCs as evidenced by an altered cytokine production profile of responding T cells.

Discussion

In this study, we compared, side by side, three different "immunomodulatory" DC types for their ability to steer the outcome of Ag-specific immune responses and autoimmunity. Our data reveal that all three types of DCs were able to inhibit CIA, but that their mode of action varies. The outcome of immunity after vaccination with TNF- or IL-10-modulated DCs displayed the characteristics of a Th2-mediated immune response with a skewing toward Ag-specific IgG1 Abs and a T cell response that biased toward the production of IL-5 and IL-10, as compared with the immune response that was induced by stimulation with LPS-activated DCs. Dex-modulated DCs did not induce a pronounced IL-5 response and no significant alteration in the Ag-specific IgG2a-IgG1 ratio (Fig. 5). This finding is in line with observations using human monocyte-derived DCs that were triggered with CD40 in the presence of Dex (17). Such Dex-modulated DCs have been shown to be poor stimulators of Th1-type responses and able to induce a state of hyporesponsiveness in Th1 cells, suggesting that these DCs contribute to active suppression of Th1-type immunity. Our data confirm and extend these findings as they indicate that not only Th1-type responses are inhibited, but also that they can be used to inhibit the induction of Th1-associated autoimmune diseases.

Our data did not reveal the presence of Foxp3^{+} regulatory T cells (Treg) as we did not observe the emergence of CD4^{+}CD25^{+} Foxp3-expressing T cells following vaccination with any of the DC types studied (data not shown). Furthermore, T cells induced after stimulation with these tolerogenic DCs were not able to suppress T cell responses in vitro, as measured in a conventional T cell suppressor assay (data not shown). We, therefore, consider it likely that TNF- as well as IL-10-modulated DCs skewed the immune response to a Th2 or, respectively, Th3 phenotype. Although we did not directly measure T cell responses in an autoimmune model (i.e., the CIA model), these data are also in line with a study performed by Menges et al. (8) in which prevention of the Th1-associated autoimmune disease experimental autoimmune encephalomyelitis via vaccination with TNF-modulated,

myelin oligodendrocyte glycoprotein peptide-pulsed DCs was associated with an increased expression of IL-10-producing myelin oligodendrocyte glycoprotein-specific T cells. Conversely, a recent study described that vaccination with TNF-modulated DCs led to the expansion of Ag-specific, Foxp3^{+} regulatory T cells (23). However, in this case, the TNF-modulated DCs were loaded with thyroglobulin, a soluble self-protein produced by the thyroid gland which is abundantly present in serum. It is, therefore, conceivable that in this case the TNF-modulated DCs expanded the "naturally occurring" CD4^{+}CD25^{+} Ag-directed T cells, whereas in the case of OVA or bovine CII, a "novel" T cell response is induced from naive precursor cells.

The immune system is constantly facing new Ags and proteins. These Ags can be located either intracellularly or extracellularly, placing different demands on the immune system. Likewise, no harmful immune response should be generated to many novel Ags as they are normal constituents of, for example air, food, or derived from the fetus. In general, the proper (e.g., Th1-, Th2-, Treg-) type of immune reaction should be generated quickly to avoid unwanted immune-mediated pathology and to combat a pathogen in the most efficient way. For this reason, it is not surprising that the information about the microbial threat, combined with signals from the surrounding tissue, is translated rapidly by the DC into a "molecular response mode," which is reflected by their phenotypic and functional plasticity. In the absence of any inflammation or pathogenic elements, most DCs in peripheral tissues and lymphoid organs have a resting, immature phenotype characterized by high endocytic capacity and low surface expression of MHC and costimulatory molecules. However, upon interaction with microbial ligands, proinflammatory cytokines, or CD40L, DCs rapidly acquire an activated phenotype. These mature DCs have a very efficient T cell priming ability as a consequence of up-regulation of MHC and costimulatory molecules on their cell surface. This plasticity is also reflected by our finding that the different DC types clearly express a different phenotype after the different treatments as measured by the limited set of markers analyzed. For example, IL-12p40 production was unaffected in Dex-modulated DCs, but reduced in TNF- and IL-10-modulated DCs. It cannot be ruled out that IL-12p70 is produced in vivo after adoptive transfer, although we could not detect significant amounts of IL-12p70 produced by the three modulated DC types in vitro (data not shown). Therefore, it is conceivable that the relatively high levels of IL-12p40 produced by Dex-modulated DCs is instrumental in the inhibition of Th1 immunity as IL-12p40 has been reported as an IL-12p70 antagonist (24, 25). IL-6 was predominantly produced by LPS-stimulated DCs, which is of interest given the recent observations implicating a role for IL-6 in setting the balance between Th17 and Treg cell development (26, 27), as well as its ability to inhibit the action of Treg cells (28).

Intriguingly, expression of the Notch-ligand Delta 4, which was rapidly induced after treatment by LPS, was completely inhibited through the action of Dex, and clearly reduced after exposure to IL-10. Delta 4 is a member of the Notch family and a ligand for the Notch receptors 1–4. It has been shown that Delta 4 expression on APCs is instrumental in the production of IFN-γ by CD4^{+} T cells, whereas expression of Jagged on APCs is associated with IL-4 and IL-5 production (19). For this reasons, it is thought that Delta 4 induces Th cells with a Th1 phenotype, whereas Jagged constitutes an instructive signal for Th2 cell differentiation. The lack of expression of Delta 4 in TNF-modulated DCs and the diminished expression of this Notch ligand in IL-10-modulated DCs as compared with DCs that have been matured in the absence of IL-10 is in line with the reduced induction of Th1-associated immunity as measured both by the altered IgG1-IgG2a ratio and the

diminished proportion of T cells producing IFN-γ. Likewise, a small but reproducible increase in PD-L1 expression was observed on LPS-treated DC, pre-exposed to IL-10, pointing to an "anti-inflammatory phenotype" of the latter DCs as PD-1 triggering of T cells can lead to their inactivation (29).

Our results indicate that vaccination with different types of DCs can have similar effects on the clinical outcome of autoimmune disease. However, the pathways responsible for these similar effects might differ and do not necessarily involve the induction of tolerance, but could also engage immune "deviation." For this reason, caution should be taken when adopting a DC vaccination protocol that has been successfully used to inhibit certain autoimmune diseases to other forms of autoimmunity as this might lead, depending on the pathogenic mechanisms underlying disease induction/progression, to exacerbation of the disease.

Whether the use of Ag-specific immune intervention protocols will be feasible for the successful treatment or modulation of RA remains to be established. Currently, no Ags that are casually related to the induction and/or maintenance of RA are known. However, it has been recently shown that Abs against citrullinated proteins (ACPAs) predate the onset of RA by several years (30, 31). Moreover, we have shown that the presence of ACPAs in patients diagnosed as having undifferentiated polyarthritis is highly predictive for the progression of RA (32). These data imply that testing for ACPAs allows the accurate identification of individuals who are at risk for the development of RA during the preclinical phase of the disease. Such identification of individuals "at-risk" might allow Ag-specific interventions before full-blown RA become established, thereby permitting implementation of primary precautions in an Ag-specific manner.

Disclosures
The authors have no financial conflict of interest.

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